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MICROSATELLITE LETTERS

Conserved microsatellite markers of high cross-species utility for flying, ground and tree squirrels

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Abstract Many squirrel species around the world are threatened by forest loss and fragmentation. To facilitate studies of squirrel biodiversity, particularly of flying squirrels in Southeast Asia, we identified *Hylopetes*, *Menetes*, *Glaucomys* and *Sciurus* squirrel microsatellite sequences with homologs in a second squirrel species (*Spermophilus tridecemlineatus*), designed 40 consensus markers and tested three squirrel species. When tested in four individuals per species, 26 markers were variable in *Hylopetes phayrei*, 25 markers in *H. lepidus* and 25 markers in *Menetes berdmorei*. Eleven markers were selected from 14 that were polymorphic in all three species. Cross-species utility was confirmed for these 11 markers in seven additional squirrel species, including: the flying squirrels *H. phayrei*, *H. lepidus*, *H. spadiceus* and *Petaurista petaurista*; a ground squirrel, *M. berdmorei*; and the tree squirrels, *Callosciurus caniceps* and *C. finlaysoni*. The other markers that were variable in one or multiple species are also useful for those specific species.

Keywords Enhanced cross-species utility · Sciuridae · Simple tandem repeat (STR) · Squirrel · Thailand

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Many forest taxa, including squirrels, are becoming increasingly endangered due to the effects of forest loss and fragmentation (Sodhi et al. 2010). This is particularly true in Southeast Asia that has the highest rates of forest loss worldwide (Sodhi et al. 2004). In Southeast Asia, squirrel species vary in IUCN status from Critically Endangered (e.g. *Biswamoyopterus biswasi* (the Namdapha Flying Squirrel) in northeast India) to Least Concern (http://www.iucnredlist.org/details/2816/0). However the risks are certainly underestimated as true species diversity remains unknown and many taxa are classified by the IUCN as Data Deficient. It is important to note that even though some species are listed as Least Concern they actually face significant threats. For example, two of our study species: *Hylopetes lepidus* (the Grey-cheeked Flying Squirrel); and *Hylopetes phayrei* (Phayre's Flying Squirrel), are hunted extensively and sold in food markets throughout Thailand (SJ personal observation), despite being officially protected under the Preservation and Protection of Wildlife Act of B.E. 2535 (1992) (http://www.forest.go.th). Here we developed conserved microsatellite markers to facilitate conservation genetics studies in a broad range of squirrel taxa but particularly flying squirrels of the genus *Hylopetes* from Indochina.

Genomic DNA was extracted from ear clips using a phenol–chloroform extraction method. Microsatellite-enriched genomic libraries were constructed separately following Armour et al. (1994) using one adult female from each of three species: the flying squirrels: *H. phayrei* and *H. lepidus*; and the Indochinese Ground Squirrel, *Menetes berdmorei*, all sampled in Thailand. From these we generated 89, 85 and 91 unique microsatellite sequences (EMBL accession numbers LN650709–650973), respectively. Microsatellite sequences were available from GenBank for five other squirrel species: *Glaucomys sabrinus, G. volans, Sciurus lis, S. niger* and *S. vulgaris*.

Table 1 Characterization of eleven conserved microsatellite markers in three squirrel species

Locus	Locus source species, EMBL accession number and clone name	Repeat motif.	[a]Primer sequence 5′–3′	Primer *Tm* (°C)	PCR *Ta* (°C)	Exp. allele size (bp)	Species and n tested	Obs. allele size range in species	*K*	H_O	H_E	*p*HWE	Estimated null allele frequency
Hlep26	*Hylopetes lepidus;* LN650734, PHS33_46D09	(CA)16	F:[6FAM]AAGGTGGTCATCAGTTTCATTG	58.96	59	341	HL.8	284–294	6	0.88	0.84	0.43	ND
			R:AGTGAATCAGAGTGAGCGATG	58.04			HP 28	284–291	6	0.64	0.63	0.75	−0.02
							MB 28	307–336	10	0.75	0.84	0.24	0.05
Hlep59	*Hylopetes lepidus;* LN650767, PHS33_46H07	(GT)11 (GA)21	F:[6FAM]AATAAATGCTGCTGAAACAAACTC	58.93	59	297	HL 8	272–300	5	0.63	0.71	0.61	ND
			R:GCTGTGCATTAGCCTCAAAG	58.68			HP 28	277–300	11	0.73	0.88	0.07	0.09
							MB 28	313–329	7	0.82	0.82	0.30	−0.01
Hlep72	*Hylopetes lepidus*; LN650780, PHS33_53C10	(GT)14	F:[6FAM]GCCAAACCACTGCTATCC	56.60	55	243	HL 8	233–257	5	0.38	0.71	0.02	ND
			R:GKGRTAATCCTAGCCACTTG	54.83			HP 28	235–243	6	0.75	0.71	0.96	−0.04
				(t,g)			MB 28	234–261	11	0.89	0.84	0.93	−0.05
Hlep80	*Hylopetes lepidus;* LN650788, PHS33_53F04	(TG)20	F:[HEX]AATACTKAATGSAATGTGTGCAA	59.40	55	289	HL 8	288–290	3	0.50	0.66	0.38	ND
			R:CTTCCATCAGCTCGGTCA	(g,g)			HP 28	270–295	14	0.89	0.88	0.46	−0.02
				58.36			MB 28	268–281	8	0.68	0.84	0.08	0.09
Hph17	*Hylopetes phayrei;* LN650810, WP11_43C04	(CT)2 (TA)2 (CA)11 (T)4	F:[HEX]GAGTCCAKKGCCAAAKGAGA	62.50	59	205	HL 8	172–176	4	0.50	0.44	1.00	ND
			R:AGCCTGGAAACTAGGACAGTG	(t,g,g)			HP 28	158–185	15	0.86	0.91	0.76	0.02
				58.47			MB 28	157–168	6	0.61	0.58	0.50	−0.02
Hph46	*Hylopetes phayrei;* LN650839, WP11_43G11	(GT)15	F:[6FAM]GGAATAAAGGAACTCAAATGCTTC	59.55	59	170	HL 8	150–153	3	0.50	0.69	0.70	ND
			R:CCTTGTAAGTATCCTGCAATTGTG	59.95			HP 28	138–152	10	0.54	0.83	0.00*	0.21
							MB 28	148–164	5	0.79	0.77	0.89	−0.03
Hph55	*Hylopetes phayrei;* LN650848, WP11_43H11	(AC)18	F:[6FAM]CACTCTGGACCTGCCACAT	59.68	59	174	HL 8	159–169	5	0.63	0.53	1.00	ND
			R:GATGCTGAGGTTGGAATTTCTT	59.60			HP 28	167–172	15	0.86	0.90	0.08	0.01
							MB 28	168–176	5	0.71	0.68	0.37	−0.03
Hph89	*Hylopetes phayrei;* LN650882, WP11_51H12	(AC)4 (A)3 (CA)9 (AC)2	F:[HEX]GTTCACAGGTATGCTAATGCTG	57.48	55	173	HL 8	175–181	4	0.88	0.68	0.48	ND
			R:TATCAGATTCTGAAGCAGAGG	54.77			HP 28	166–179	5	0.21	0.23	0.24	0.08
							MB 28	180–201	7	0.89	0.78	0.56	−0.08
GLSA22	*Glaucomys sabrinus;* FJ755453	(CA)15	F:[HEX]CCTGARWATRATGCATGTGG	59.92	59	179	HL 8	174–184	5	0.88	0.83	0.13	ND
			R:AGAGTAGGCTGTTCCTTTGAGG	(a,a,g)			HP 28	179–189	8	0.82	0.86	0.06	0.01
				59.05			MB 28	162–221	19	0.52	0.96	0.00*	0.28
GLSA48	*Glaucomys sabrinus;* FJ755454	(CA)10 (CG)6 (CA)6	F:[6FAM]CTGCTGCAGYRACTTCCTGT	59.21	55	224	HL 8	209–213	4	0.88	0.74	1.00	ND
			R:GAGTGGGCTCTCAGGTTGA	(t,g)			HP 28	201–213	7	0.71	0.69	0.78	−0.03
				58.90			MB 28	211–215	5	0.54	0.56	0.69	0.00

Table 1 continued

Locus	Locus source species, EMBL accession number and clone name	Repeat motif.	[a]Primer sequence 5′–3′	Primer *Tm* (°C)	PCR *Ta* (°C)	Exp. allele size (bp)	Species and n tested	Obs. allele size range in species	*K*	H_O	H_E	*p*HWE	Estimated null allele frequency
ScnFO35	*Spermophilus tridecemlineatus;* FJ477952	(CA)12 A (CA)3	F:[6FAM]GATGGACATCTGAAATAGTGAGA R:ACACTGGGCTAAACAACAAA	55.90 55.83	55	180	HL 8 HP 28 MB 28	168–176 157–176 159–168	6 9 9	0.50 0.93 0.61	0.86 0.87 0.80	0.11 0.42 0.03	ND −0.04 0.13

Tm, melting temperature for the primer sequence that matches the stated source species estimated using PRIMER3 v0.4.0 (where the lower case letters in brackets indicate the bases present in the source species at each respective ambiguity site); *Ta*, PCR annealing temperature; Species tested: HL = *Hylopetes lepidus*, HP = *Hylopetes phayrei*, MB = *Menetes berdmorei*; n, number of individuals genotyped; *K*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *p*HWE, probability of Hardy–Weinberg equilibrium

* $p < 0.01$

[a] Primer sequence designed from the consensus sequence of the clone and the homologue from *Spermophilus tridecemlineatus*

We followed the approach of Dawson et al. (2010) to identify conserved microsatellite sequences and create markers of high cross-species utility. We created a consensus sequence by aligning the newly isolated squirrel microsatellite sequences and/or other online squirrel sequences (Hylopetes/Menetes/Glaucomys/Sciurus) against their homologue in the thirteen-lined ground squirrel genome (*Spermophilus tridecemlineatus*; http://www.ensembl.org/Spermophilus_tridecemlineatus/index.html). Primer sets were designed for 40 microsatellite loci based on these consensus sequences using PRIMER3 v0.4.0 (avoiding bases mismatching between species, when possible). Each primer set matched *S. tridecemlineatus* and one of the other eight squirrel species cited above (the "source" species) with a maximum of three degenerate bases per primer (three per primer set) and a maximum of one base mismatching *S. tridecemlineatus*. The optimal difference between the forward and reverse primer melting temperatures was set to 0.5 °C (maximum 4 °C; Table 1).

The 40 conserved microsatellite markers were tested in our primary study species *H. phayrei*, *H. lepidus* and *M. berdmorei* (using four individuals per species). Twenty-six markers were variable in *H. phayrei*, 25 markers in *H. lepidus* and 25 markers in *M. berdmorei* (Supplementary Table 2). Fourteen loci amplified and were polymorphic in all three species (Supplementary Table 2). Primer sets designed from comparisons of the thirteen-lined ground squirrel with the flying squirrels (*Hylopetes*) amplified in more species than those designed from comparisons between the two ground squirrel species, *M. berdmorei* and *S. tridecemlineatus* (Supplementary Tables 1 and 2). This is likely due to the greater phylogenetic distance between *Hylopetes*/*Glaucomys*/*Sciurus* genera and *S. tridecemlineatus* than between *M. berdmorei* and *S. tridecemlineatus* (Mercer and Roth 2003), resulting in more highly conserved primers for the former. The 14 microsatellite loci were evaluated in a greater number of individuals: *H. phayrei* (28 individuals from Mae Rim, Thailand); *H. lepidus* (eight individuals from Phu Huay Sing, Thailand); and *M. berdmorei* (28 individuals from Wapipathum, Thailand). PCR reactions were carried out in a DNA Engine thermal cycler (MJ Research) in 2 µl volumes containing 10 ng genomic DNA, 1 µl Qiagen Multiplex PCR Master Mix (Qiagen Inc.) and primers (0.2 µM). Initial denaturation for 15 min at 95 °C was followed by 34 cycles of 30 s at 94 °C, 90 s at the optimal annealing temperature (Table 1) and 60 s at 72 °C with a final extension step of 30 min at 60 °C. PCR products were run on a 48-capillary ABI3730 DNA Analyzer using prism set D and a ROX size standard and the alleles sized with GENEMAPPER ver. 3.7 (Applied Biosystems). Observed heterozygosity (H_O), expected heterozygosity (H_E) and estimated null allele frequencies were calculated using CERVUS v3.0.3. Deviations from Hardy–Weinberg equilibrium and tests for linkage

Table 2 Cross-species utility of conserved squirrel microsatellite loci in four additional squirrel species (two flying squirrels and two *Callosciurus* tree squirrels)

Species tested	Locus	Hlep26	Hlep59	Hlep72	Hlep80	Hph17	Hph46	Hph55	Hph89	GLSA22	GLSA48	ScnFO35
Expected allele size (bp)		341	297	243	289	205	170	174	173	179	224	180
Hylopetes spadiceus	N tested	13	13	13	13	13	13	13	13	13	13	13
	N amp.	13	13	13	12	13	13	13	13	0	13	13
	Size (bp)	278–294	282–313	233–249	259–291	159–180	137–156	162–178	174–184	–	188–215	222–247
	K	7	9	8	10	5	6	7	5	0	9	9
Petaurista petaurista	N tested	2	2	2	2	2	2	2	2	2	2	2
	N amp.	2	2	2	1	2	2	2	2	0	2	1
	Size (bp)	288, 294, 296	285, 289, 293	239, 241, 253	289	162–180	140, 152, 157	155, 166, 182	172, 177, 179	–	209–225	170,176
	K	3	3	3	1	4	3	3	3	0	4	2
Callosciurus caniceps	N tested	19	19	19	19	19	19	19	19	19	19	19
	N amp.	19	19	6	0	19	19	19	19	2	19	1
	Size (bp)	322–347	284–335	226–238	–	162–174	148–159	169–182	182–207	173, 177, 179	204–225	162
	K	12	12	4	0	7	6	8	12	3	11	1
Callosciurus finlaysoni	N tested	24	24	24	24	24	24	24	24	24	24	24
	N amp.	17	24	24	13	24	24	24	23	4	24	15
	Size (bp)	286–339	290–337	200–231	293	150–174	132–159	166–184	194–218	175–183	198–207	155–184
	K	9	14	7	1	10	8	8	14	4	5	8

N tested, number of individuals for which PCR was attempted for each species and locus; N amp, number of individuals for which amplification products were detected; Size (bp), observed allele size range (base pairs); K, number of alleles observed

disequilibrium were calculated using GENEPOP v.4.2 (http://genepop.curtin.edu.au/).

Three loci were excluded due to low variability in *M. berdmorei,* one of the three test species (*Hph14*, *Hph54* and *Hlep05*; Supplementary Table 1). For the remaining 11 loci, the number of alleles per locus across the three species ranged from three to 19 (Table 1). Heterozygotes were observed in males and females for each species suggesting none of the 11 loci were sex-linked. Observed and expected heterozygosities ranged from 0.21 to 0.93 and from 0.23 to 0.96, respectively (Table 1). Two loci deviated from Hardy–Weinberg equilibrium in some species ($p < 0.01$, *GLSA22* and *Hph46*, Table 1), which may be due to null alleles. There was no evidence of linkage disequilibrium between any groups of loci in any species.

The majority of the 11 markers could be amplified and were polymorphic in other squirrel species; ten loci in the flying squirrels *Petaurista petaurista* and *Hylopetes spadiceus* and seven loci in the tree squirrels *Callosciurus caniceps* and *C. finlaysoni* (Table 2). These markers will therefore be useful for conservation studies in a wide range of squirrel taxa.

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